PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : A61K 47/48, C12N 9/38 — A1

(11) International Publication Number: WO 99/06072

(43) International Publication Date: 11 February 1999 (11.02.99)

(21) International Application Number: PCT/US98/15433

(22) International Filing Date: 24 July 1998 (24.07.98)

(30) Priority Data:
60/054,285 30 July 1997 (30.07.97) US

(71) Applicant *(for all designated States except US)*: BOEHRINGER MANNHEIM CORPORATION [US/US]; 9115 Hague Road, Indianapolis, IN 46250–0457 (US).

(72) Inventor; and
(75) Inventor/Applicant *(for US only)*: POWELL, Michael, J. [US/US]; 1024 Ocho Rios Drive, Danville, CA 94526–4820 (US).

(74) Agents: SCHIFF, J., Michael et al.; Morrison & Foerster LLP, 755 Page Mill Road, Palo Alto, CA 94304–1018 (US).

(81) Designated States: CA, JP, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: CYCLIZED PRODRUGS

(57) Abstract

Cyclized prodrugs of this invention are covalently cross–linked so as to inhibit their ability to perform the usual biological or metabolic function of therapeutic benefit. Either the polypeptide backbone of the enzyme or the cross link itself contains a cleavable site. In an environment where the enzyme specific for the cleavable site is expressed, the cross–linked prodrug is released from its inhibited state and again becomes capable of exerting its therapeutic effect.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

CYCLIZED PRODRUGS

REFERENCE TO RELATED APPLICATIONS

This application claims the priority benefit of U.S. Provisional Patent Application 60/054,285, filed July 30, 1997, pending. For purposes of prosecution in the U.S., the priority application is hereby incorporated herein by reference in its entirety.

FIELD OF THE INVENTION

This invention relates to compounds and compositions of therapeutic value in human and veterinary clinical care. Specifically, proteins with a desirable biological effect are cyclized by an intramolecular cross-link so that they may be administered in an inert form and become activated near a target area.

BACKGROUND OF THE INVENTION

In the past, various synthetic and natural antigenic polypeptides and polypeptide fragments have been conjugated to high molecular weight protein carriers such as latex functionalized SEPHAROSE™ (Pharmacia, Inc.), tetanus toxoid, keyhole limpet hemocyanin, agarose and cellulose to detectable labels such as fluorophores, and to chemotherapeutic agents using bifunctional cross-linking agents. U.S. Patent 4,493,795 and PCT publication WO 90/05749 (published May 31, 1990) are exemplary. Such cross-linking agents have also been used to attach bioactive or cytotoxic agents, dyes, radioactive compounds and the like to antibody molecules. U.S. Patent No. 4,671,958 is exemplary. Antibodies have been linked together using such agents. See Chen, *Res. Virol. 141*:337-42 (1990). Cross-linking agents have also found use for modifying bioactive and therapeutically useful polypeptides by conjugation with polymers such as polyethylene glycol to enhance pharmacokinetic properties. U.S. Patent Nos. 5,166,322, 4,179,337 and 4,766,106 are exemplary.

β-Galactosidase is a tetrameric protein with a monomer molecular weight of approximately 116,000 Daltons. The monomer is composed of 1023 amino acids. Intracistronic complementation is the known phenomenon whereby individually inactive peptide fragments of the enzyme spontaneously associate to form an active β-galactosidase protein. Among the first galactosidase complementation pairs investigated in depth was the MI5/CNBr2 system described by Langley and Zabin, *Biochemistry 15*:4866 (1976). M 15 is a deletion mutant of β-galactosidase lacking amino acids 11-41. The CNBr2 peptide consists of amino acids 3-92 of β-galactosidase and is prepared from cyanogen bromide cleavage of the intact enzyme. When MI5 and CNBr2, which are

individually inactive, are incubated together under appropriate conditions, the two peptides complement or associate with each other to form fully active, tetrameric β-galactosidase. In this system, CNBr2, the N-terminal peptide, is referred to as the α-enzyme donor. MI5, which has the N-terminal deletion, is referred to as the α-enzyme acceptor. The general phenomenon which uses the reassociation of the domains of β-galactosidase to form active β-galactosidase from inactive fragments is referred to as complementation. Other combinations of α-enzyme donors and α-enzyme acceptors have been described. See Zabin, *Mol. and Cellular Biochem 49*:84 (1982). Each is a variant derived from the natural β-galactosidase sequence.

Complementation of a C-terminal peptide and corresponding C-terminal deletion protein has also been described. An example of this phenomenon, known as omega-complementation, is X-90, a β-galactosidase deletion variant lacking 10 amino acids at the C-terminus and CNBr24, a peptide comprising amino acids 990-1021 of β-galactosidase. As in the case of α-complementation, ω-enzyme donor polypeptides and ω-enzyme acceptor proteins are inactive but reassociate to form enzymatically active tetramer. See Welphy, *Biochem. Biophys. Res. Comm. 93*:223 (1980).

β-galactosidase complementation activity has been exploited to produce sensitive quantitative assays for both high and low molecular weight analytes. U.S. Patent Nos. 5,362,625 and 4,708,929 disclose, inter alia, a variety of enzyme donor and enzyme acceptor polypeptide compositions for use in antibody and receptor binding assays. The enzyme donors and enzyme acceptors are generated by means of recombinant DNA or polypeptide synthesis techniques familiar to skilled artisans.

These approaches allow great flexibility and control over the design of enzyme donor and enzyme acceptor molecules. The use of genetic engineering techniques allows the sequence and length of the enzyme donor and enzyme acceptor polypeptides to be modified to maximize assay performance and reagent stability. Enzyme donors optimized for chemical coupling to analyte and enzyme donors genetically fused to analyte peptides or proteins have been described, and immunoassays using these compositions are commercially available. See Henderson, *Clin. Chem. 32*:1637 (1986); Khanna, *Amer. Clin. Lab 8*:14 (1989) and Coty, *J. Clin. Immunoassay 17*:144 (1994).

The following patent applications relate to reagents and methods useful for measuring analytes using cross-linked derivatives of enzyme donor fragments of β-galactosidase: U.S. non-provisional patent applications 08/592,013, filed January 26, 1996, pending; 08/592,029, filed January 26, 1996, pending; 08/592,015, filed January 26, 1996, abandoned; and [S/N pending, attorney docket 33746-20005.01], filed June 26, 1997, pending; and International patent applications designating the U.S. PCT/US97/01129; PCT/US97/00883; and PCT/US97/01130; all filed January 23, 1997, pending.

SUMMARY OF THE INVENTION

This invention provides potentially therapeutic compounds, compositions and methods for their preparation and use. The embodiments of the invention are based on the observation that activity of proteins can be greatly but reversibly inhibited by introducting a cross-link into the peptide structure. A cyclized prodrug having a cross-linking moiety can be administered to a subject in a relatively inert form, preserving the activity and reducing side effects. Where the cyclized prodrug further comprises an enzymatically cleavable site that leads to decyclization and recovery of therapeutic activity, the prodrug can later be activated.

A powerful feature of certain embodiments of this technology is that the enzyme cleavable moiety can be designed so that it is cleavable only by a specific activating enzyme at the cells or tissue site that are the object of treatment. Thus, a prodrug of this invention will be preferentially activated near inflammatory tissue if the cleavable moiety is a substrate for an enzyme expressed at an elevated level during inflammation. A prodrug of this invention will be preferentially activated near an infected cell if the cleavable moiety is a substrate for an enzyme expressed by the infectious virus or bacterium. Even when administered systemically or across a mucosal surface, activation may occur in a targeted fashion, with prodrug in unaffected tissue being left in the inert state.

Included amongst the embodiments of this invention is a cross-linked prodrug, having an inserted enzyme recognition site in a polypeptide of the prodrug and at least one covalent intrachain cross-link between amino acid side chains of the polypeptide, wherein the cross-linked prodrug is converted to a therapeutically more effective form upon cleavage of the enzyme recognition site. The activating enzyme may be a viral or bacterial protease, a protease that is expressed at elevated levels at sites of inflammation or malignancy, or a protease expressed by cells undergoing programmed cell death.

Also embodied in this invention is a cross-linked prodrug, having an inserted enzyme recognition site in a polypeptide of the prodrug and at least one cross-linking moiety covalently bonded between amino acid side chains of the polypeptide, wherein the cross-linking moiety comprises an enzymatically cleavable site, and wherein the cross-linked prodrug is converted to a therapeutically more effective form upon cleavage of the enzymatically cleavable site. Exemplary activating enzymes that cleave cross-linking moities include endoglycosidases and nucleases.

In the activated form, the prodrugs of this invention are preferably at least 10 times more effective than the prodrug, and may have any desired biological activity, including but not limited to the suppression of unwanted inflammatory or immunological activity, the promotion of growth, or a direct cytotoxic effect on the target cells.

Also embodied in the invention are methods of preparing the prodrugs of this invention, comprising cyclizing a cytotoxin or an immunotoxin by cross-linking two amino acids in the polypeptide.

Additional embodiments include pharmaceutical compositions containing the prodrugs of this invention, and methods for treating a subject for a disease associated with increased local expression of an enzyme, comprising administering to the subject a cross-linked prodrug, wherein the locally expressed enzyme is specific for the enzyme recognition site or the enzyme cleavable site of the prodrug.

Other aspects and advantages of the present invention will be apparent upon consideration of the following detailed description of the invention, including illustrative examples of the practice thereof.

BRIEF DESCRIPTION OF THE DRAWINGS

The present invention will be better understood by reference to the following detailed description of the invention when considered in combination with the drawings that form part of the specification, wherein:

Figure 1 is a particular synthetic scheme for preparing N-(2-trimethylsiloxyethyl)-maleimide.

Figure 2 is a particular synthetic scheme for preparing 4-maleimidobutyraldehyde.

Figure 3 is a particular synthetic scheme for preparing 1,7-bismaleimido-4-O-(tetraacetyl-β-D-galactopyranosyl)-5-oxaheptane and 1,7-bis-(3'-methoxysuccinimido)-4-O-(β-D-galactopyranosyl)-5-oxaheptane.

Figure 4 is a sequence listing for several exemplary cross-linked enzyme components, incorporating a core enzyme donor sequence of β-galactosidase, an enzyme recognition sequence in the N-terminal direction, and a reactive amino acid (i.e., cysteine) linked to the N-terminal end via a short spacer sequence comprising a glycine repeat. The first enzyme component (SEQ. ID NO:9) contains the ICE protease recognition sequence YVAD (SEQ. ID NO:10) inside the artificial sequence CGGGYVADG (SEQ. ID NO:11), which in turn is linked onto the enzyme donor core. The second enzyme component (SEQ. ID NO:12) contains the Caspase recognition sequence DEVD (SEQ. ID NO:13) inside the artificial sequence CGGGDEVDG (SEQ. ID NO:14), which in turn is linked onto the enzyme donor core. The third enzyme component (SEQ. ID NO:15) contains the IgA protease recognition sequence TPPTPSPS (SEQ. ID NO: 16) inside the artificial sequence CKGGGTPPTPSPS (SEQ. ID NO:17), which in turn is linked onto the enzyme donor core. The enzyme components are cyclized through the added N-terminal Cys and the Cys imbedded near the C-terminal of the core. In the presence of the corresponding analyte enzyme, the recognition sequence is cleaved and the component is linearized, allowing it to assemble with an enzyme acceptor and provide an assay signal.

Figure 5 is a two-panel chart showing assay results of several separated fractions of a cross-linked enzyme component having the ICE protease recognition sequence YVAD (SEQ. ID NO:10). The upper panel is a chart of absorption data for the fractions used for an assay at various

dilutions of the protease. The lower panel compares the rate of the reaction in the presence and absence of the protease.

Figure 6 is a listing of the S-peptide (SEQ. ID NO:18) and S-protein (SEQ. ID NO:19) components of ribonuclease, which complement to create enzymatic activity. A cyclized enzyme component of this invention is made from either subunit by inserting an intramolecular cross-link; for example, between the lysine residues at positions 1 and 7 of the S-peptide.

Figure 7 is a map of the cleavage sites of IgA-specific protease for various pathogenic bacteria in the hinge region of human IgA1 (PSTPPTPSPSTPPTPSPSC; SEQ. ID NO:20). The hinge region of human IgA2 (PPPPPC; SEQ. ID NO:21) lacks the tandem repeat TPPTPSPS (SEQ. ID NO:16) and is not cleaved by these proteases. (Figure adapted from Figure 1 of Kornfeld & Plaut, 1981, *Rev. Infect. Dis.* 3:521). The presence of IgA protease correlates with bacterial pathogenicity; hence prodrugs with the recognition sequence will preferentially activate near pathogenic strains.

DETAILED DESCRIPTION OF THE INVENTION

This invention provides cross-linked prodrugs, based on the sequence of any protein drug with therapeutic activity or capable of being assembled, cleaved, or otherwise altered into a form that exhibits therapeutic activity. Also embodied are methods of making such prodrugs, and their use in the treatment of a subject afflicted by a condition amenable to the therapeutic effect of the activated drug.

A "cyclized prodrug" or "cross-linked prodrug" of this invention is a compound comprising a polypeptide chain which has been covalently crosslinked internally or to a neighboring polypeptide chain. It has the characteristic of being able to refold or assemble so as to form a therapeutically effective compound, when a bond within the compound, usually either within a polypeptide or the cross-linking moiety, is cleaved by a catalytic molecule (usually an enzyme) in the environment to which it is administered.

An "activating enzyme" is an enzyme capable of cleaving a prodrug of this invention at the recognition site, thereby permitting it to assume the more active or therapeutic form. It may be administered to a subject in order to activate a previously administered prodrug, in order to tailor the location or timing of a more generally (perhaps systemically) administered prodrug. More typically, it is produced endogenously by a disease site. In this way, a more generally administered prodrug will become activated in a gradient centered near the desired site of action.

Following the cleavage of the enzyme recognition site, the prodrug reassembles internally by unfolding or refolding, or reassembles with a second or a plurality of components in the reaction mixture, so as to form the therapeutic drug. Where internal reassembly is involved, the prodrug will contain a non-naturally occurring cross-link which is cleavable by the enzyme, or a non-naturally occurring cross-link along with a heterologous sequence in a polypeptide that is cleavable by the

enzyme. Where reassembly with a second component is involved, the component will contain a non-naturally occurring cross-link cleavable by the enzyme, or a natural or non-naturally occurring cross link along with a heterologous sequence in a polypeptide that is cleavable by the enzyme.

The nature of the recognition site for the activating enzyme depends upon the application to which the cross-linked prodrug will be put. The recognition site may comprise a peptide sequence or a nucleic acid sequence that is cleavable by an enzyme or a specific protease, nuclease or endoglycosidase by virtue of containing a protease, nuclease or glycosidase recognition sequence. Alternatively, the recognition site may comprise a substrate recognition site for a specific hydrolase enzyme such as a phosphatase, glycosidase, amidase or esterase.

Where the recognition site is a peptide sequence, it is typically contained within the polypeptide backbone of the prodrug, incorporated during peptide synthesis or by recombinant expression. Where the recognition site is for a nuclease, endoglycosidase, or other non-protease, it is typically contained within the cross-linking agent itself. The nature and assembly of these components are described in the sections that follow.

Drug prototypes

The cross-linked prodrugs of this invention are typically based on a previously known naturally occurring or reengineered protein drugs with known structure and pharmacologic effect. They comprise a polypeptide (optionally glycosylated) with amino acid residues that are cross-linked through their side chains. The cross-link may be a disulfide bond, an amide bond, or any bond that is formed by cross-linking agents with at least two linking positions.

A "peptide" is any compound formed by the linkage of two or more amino acids by amide (peptide) bonds, usually a polymer of α-amino acids in which the α-amino group of each amino acid residue (except the NH_2-terminal) is linked to the α-carboxyl group of the next residue in a linear chain. The terms peptide, polypeptide and poly(amino acid) are used synonymously herein to refer to this class of compounds without restriction as to size. The larger members of this class are referred to as proteins.

The therapeutic activity of the reassembled complex may be of any nature that is of therapeutic benefit to the subject being treated, through either a direct or inhibitory effect.

An illustrative example of therapeutically effective peptides suitable for adaptation as prodrugs of this invention include modulators of the inflammatory and immune system, particularly cytokines and peptidase cleavage products of the complement, kallikrein, and plasminogen pathways with cell modulation activity. In this family, mediators that have a sustained dampening effect on inappropriate inflammation or autoimmunity are of interest. Potential enzyme activators for this type of peptide prodrug are those likely to be present at such diseased sites. In particular, the complement, kallikrein, and plasminogen pathways and a number of related cell-surface phenomena

involve a cascade of proteolytic reactions of considerable specificity, and enzyme recognition sequences for the prodrug could be chosen accordingly.

Another example is growth factors and hormones that positively stimulate the regrowth of a depleted tissue. Included are factors that stimulate the hematopoietic system and cells that cause regeneration of vital organs such as the liver. Considerable effort has been directed towards identifying tissue-specific growth factors for pluripotent cells in a number of organs. The techniques of this invention provide an alternative approach where such tissue-specific factors have not been identified. For example, a relatively non-specific growth factor such as EGF could be provided in a prodrug form with an enzyme recognition site specific for an enzyme produced exclusively by the tissue. The prodrug would then be preferentially activated in the desired location. Thus, the specificity is supplied not by a specificity of the effector component of the growth factor for the tissue of interest, but by an enzyme produced by the tissue.

Yet another example of therapeutically effective peptides suitable for adaptation as prodrugs are pharmaceutically important enzymes. Since enzymes are involved in the activation of complement, kallikrein, and plasminogen pathways and important cell-surface regulatory effects, enzyme prodrugs may be useful for enhancement or dampening of such pathways. In addition, protease prodrugs can be used for the purposes of drug cascade, where the cyclized protease is activated by a locally produced enzyme, and then cleaves other prodrugs in the administered composition with therapeutic effect.

Of particular interest in the treatment of certain diseases are prototype drugs that are directly cytotoxic to a neighboring cell. Such effectors are desired in the treatment of neoplasms, and virally or bacterially infected tissue.

Cytotoxic effector peptides include bacterial toxins, such as cholera toxin and cholera toxin B subunit, *E. coli* heat-labile enterotoxin and its B subunit, *Bordetella pertussis* toxin and the subunits S2, S3, S4, and S5 (in any combination), diphtheria toxin and the β toxin fragment, shiga and shiga-like toxins, staphylococcal α-hemolysin, vibrio thermostable direct hemolysin, alpha-sarcin, ricin, and abrin.

Other cytotoxic effector peptides are RNases, such as colicins; T1 ribonucleases (including fungal ribonucleases); T2 ribonucleases (both plant style and seed RNases and ribosome-inactivating proteins). Also of interest are members of the RNase A superfamily, including RNase A, seminal RNase, RNase dimers, eosinophil-derived neurotoxin, eosinophil cationic protein, onconase, frog ribonucleases, and angiogenin. Also contemplated for use in this invention are RNases that are engineered to be cell-type selective by coupling to ligands for cell-surface receptors (cytotoxic ribonuclease chimeras). For a critical review of RNase chimeras, the reader is referred to Youle et al., 1993, *Crit. Rev. Ther. Drug Carrier Syst.* 10:1-18.

Pseudomonas exotoxin A is a 66 kDa protein toxin that kills eukaryotic cells having specific cell surface receptors for the toxin. The toxin enters the cell by receptor-mediated endocytosis, and a 37 kDa C-terminal fragment is translocated into the cytosol, where it catalyzes the irreversible

ADP-ribosylation of elongation factor 2 (EF-2), leading to cessation of protein synthesis and cell death. The 3D structure of the toxin has been determined, and one or more functions has been assigned to each structural domain. Domain Ia (amino acids 1-252) is responsible for cell recognition and binding. Domain III (amino acids 405-613) contains the ADP-ribosylating activity. No role for domain Ib (amino acids 365-404) has been determined. Domain II (amino acids 347-364) is described as being sufficient for transport into the cytosol. For the development of therapeutic chimeras, Domain Ia can be replaced with targeting moieties, such as ligands for receptors such as TGFα and IL-2. The translocated 37 kDa fragment contains a C-terminal sequence that directs the toxin to the appropriate intracellular compartment for translocation into the cytosol. In the native toxin, the sequence is REDLK (SEQ. ID NO:23), but this can be substituted with KDEL (SEQ. ID NO:22). Pastan et al., 1996, *Breast Cancer Res. Treat.* 38:3-9. Recombinant truncated forms of Pseudumonas exotoxin are proposed for chemotherapy-resistant forms of cancer treatment. Zimmerman et al., 1997, *Cancer Immunol. Immunother.* 44:1-9.

Prior et al. (1996, *Bioconjugate Chem.* 7:23-29) have described a chimeric molecule, composed of amino acids 1-412 of *pseudomonas* exotoxin, conjoined to the extracellular ribonuclease of Bacillus amyloliquefaciens, barnase (amino acids 1-110), and then followed by terminal residues 604-613 of *pseudomonas* exotoxin. This protein is toxic to cells due to its RNase activity, which is delivered to the cell by way of the endotoxin delivery pathway.

The challenge of using cytotoxic effectors in human subjects is the difficulty in focusing the toxic effect to the cell of interest. Some of the recombinant immunotoxins and chimeras reviewed above have been engineered to preferentially bind cells with particular receptors. The techniques of this invention provide a means to provide such highly potent molecules with a second level of specificity. Specific prodrug activation followed by specific cell binding and internalization should help minimize unwanted collateral effects.

Peptidase recognition sites

Cross-linked prodrugs of this invention are cleavable via an enzyme cleavable site either within the sequence of the peptide backbone, or within the cross-linking moiety. This section describes specific amino acid sequences that can be incorporated into the peptide backbone and are cleavable by a peptidase of interest.

In most embodiments of this invention, the recognition sequence is heterologous to the naturally occurring amino acid sequence of the drug prototype. It is "inserted" into the prodrug sequence during chemical synthesis or expression of a recombinant polynucleotide with the corresponding encoding region. Assembly of the chimeric sequence is described in more detail in a later section of this disclosure.

To prevent inappropriate activation, the recognition sequence is preferably unique to the activating enzyme in comparison with other proteases that may occasionally be found at other sites

or unaffected tissue. Conversely, the activating enzyme will typically be highly specific for a particular amino acid sequence, usually at least 4 amino acids in length, such that cleavage of the prodrug will occur in a predictable fashion and not unnecessarily activated by other proteins that may be present in the reaction mixture.

Of interest are recognition sequences for viral proteases, and for proteases overexpressed during inflammation, cancer or cellular apoptosis that is associated with clinical disease states. Of particular interest are recognition sequences for HIV protease, N. gonorrhea protease, Glu-C protease, ICE protease, Caspase, and IgA protease.

Viral proteases such as HIV-1 and HIV-2 protease, coxsackie virus protease and herpes virus protease recognize specific peptide substrate sequences of the host's cellular proteins. HIV-1 protease is of particular interest because it is responsible for the proteolytic processing of the gag and gag-pol proteins to form infectious virions. See Kramer, *Science 231*:1580 (1986) and Kohl, *Proc. Natl. Acad. Sci. 85*:4686 (1988). The HIV protease recognizes and cleaves the octapeptide sequence SQNYPIVQ (SEQ ID NO:1), corresponding to the Pr55 gag p17/p24 cleavage site, and the decapeptide sequence VSFNFPQITL (SEQ ID NO:2), corresponding to the p6/PR cleavage site of the gag-pol protein. See Krausslich, *Proc. Nat. Acad. Sci. 86*:807-11 (1989). Thus, peptide sequences that are recognized and cleaved by such HIV proteases can be employed as the recognition site. See, for example, Baum, *Proc. Nat. Acad. Sci. 87*:10023-27 (1990) which discloses insertion of an HIV protease recognition site into a non-cross-linked β-galactosidase gene, and Liebig, *Proc. Nat. Acad. Sci. 88*:5979-83 (1991) which discloses fusion of a human rhinovirus proteinase with a non-cross-linked α-fragment of β-galactosidase.

Another exemplary activating enzyme is interleukin-l-β converting enzyme (ICE protease), which plays a role in apoptosis (cell death). The protease plays a role in IL-1β processing and secretion. , and is a major mediator of inflammatory disease. ICE proteases have also be implicated in the programmed cell death of neuronal cells. Schwartz et al., 1996, *Trends Neurosci.* 19:555. For a description of the structure and function of the family of ICE proteases, see Tocci, 1997, *Vitamins* & Hormones 53:27.

The ICE protease has a well-defined specificity for the sequence -X-Val-Y-Asp-Z- (SEQ ID NO:3). Cleavage of the sequence by ICE protease occurs after the Asp. The minimal and best peptide substrate sequence found heretodate to elicit action of the ICE protease enzyme appears to be Ac-Tyr-Val-Ala-Asp-NH-CH_3 (SEQ ID NO:4). See Thornberry, *Nature 356*:768-74 (1992). A potent inhibitor of ICE protease contains the sequence Ac-Tyr-Val-Ala-Asp-* (SEQ ID NO:5), where * stands for chloromethylketone. See *Nature 375*:78-81 (1995). Incorporation of the ICE protease recognition site into a synthetic cross-linked prodrug would permit the development of a simple, rapid assay for this important protease.

Another exemplary activating enzyme is Caspase, a peptidase in the same death-domain containing family as ICE protease. Overexpression of Caspase-8 induces apoptosis in an manner

that is dependent upon the cell surface marker CD95. Yuan, 1997, *Current Opin. Cell Biol.* 9:247, 1997. A potential recognition sequence for Caspase is DEVD (SEQ. ID NO:13).

Other exemplary activating enzymes are those preferentially expressed or overexpressed in neoplastic cells. Examples include cathepsin D protease in breast cancer, and prostate-specific antigen (PSA) in prostate cancer.

Other exemplary activating enzymes are proteases that are exquisitely specific for human IgA of the IgA1 subtype. The hinge region of IgA1 contains a tandem repeat of the sequence TPPTPSPS (SEQ. ID NO: 16), and this repeat is cleavable at several positions by enzymes secreted by infectious bacteria. For general reviews of the IgA family of proteases, the reader is referred to Kornfeld et al., 1981, *Rev. Infect. Dis.* 3:521; and Plaut, 1983, *Annu. Rev. Microbiol.* 37:603. IgA protease production can be used to distinguish pathogenic from harmless *Niesseriaceae:* Mulks et al., 1978, *New Engl. J. Med.* 299:973. Accordingly, prodrugs activatable by IgA protease are potentially important in treating microbial infection.

Figure 7 shows the cleavage sites of IgA-specific protease for various pathogenic bacteria in the hinge region of human IgA1 (SEQ. ID NO:20). Separate cleavage sites exist for IgA proteases for *Neisseria gonorrhoeae, Neisseria meningitidis, Hemophilus influenzae, Streptococcus pneumoniae,* and *Streptococcus sanguis.* Since IgA protease is apparently secreted by these microorganisms as a defense mechanism against IgA, and since pathogenicity correlates with IgA protease expression, the level of IgA protease is substantially high in the microenvironment of the infection. According to the techniques of this invention, a cyclized prodrug with a recognition site for IgA protease is expected to be activated preferentially at infected sites.

Cross-linking agents

Prodrugs of this invention contain at least one cross-link spanning amino acid residues.

The term "cross-link" as used in this disclosure refers to the covalent chemical attachment of a cross-linking agent between two reactive amino acid residues in the prodrug. The link may, for example, be in the form of a disulfide or peptide bond between amino acid side chains, or formed as a result of contacting the polypeptide with a multifunctional cross-linking agent. Where the cross-link is intramolecular, a "cyclized" prodrug is created.

Cross-linked prodrugs of the invention comprise sequences that contain reactive amino acid residues that permit attachment of a cross-linking agent which comprises cross-linking moieties. Reactive amino acid residues permitting attachment to such moieties are α- or ε- amino groups (e.g., lysine), α-, β- or γ-carboxyl groups (e.g., aspartic acid or glutamic acid), thiol groups (e.g., cysteine), and aromatic rings (e.g., histidine or tyrosine). Other reactive groups found on amino acids include double bonds, alcohol groups, and the like.

The cross-linking moiety is an integral part of the cross-linking agent and comprises a chemical moiety or functional group that enables the cross-linking agent to covalently bond to

reactive amino acid residues. For example, a cross-linking agent useful to covalently couple thiol groups of proteins and peptides is bis-maleimidohexane (BMH). This cross-linking agent comprises a hexamethylene moiety having maleimido cross-linking moieties attached to each end of the hexamethylene. Other exemplary cross-linking agents are described in *Chemistry of Protein Conjugation and Cross-Linking*, S.S. Wong, CRC Press, 1993.

The amine groups of reactive amino acids in the prodrug polypeptide may be cross-linked by reaction with an amino group-reactive moiety of the cross-linking agent. N-hydroxysuccinimide, dimethylsuberimidate, phenyldiisocyanate, phenyldiisothiocyanate, difluorodinitrobenzene and cyanic chloride are exemplary amino group reactive moieties suitable for use in cross-linking agents. The thiol groups of reactive amino acids may be cross-linked by reaction with a sulfhydryl-reactive moiety of the cross-linking agent. Exemplary reactive groups are S-pyridyl, maleimide and bromoacetyl moieties. The carboxyl groups of reactive amino acids may be cross-linked by reaction with carbodiimide or hydrazide moieties.

The cross-linking moieties may be homo- or heterobifunctional, such that cross-linking between the appropriate residues, preferably at or near the N-terminal and C-terminal residues of the prodrug polypeptide, is accomplished. Thus, the cross-linking agent will have two reactive groups capable of covalent chemical attachment to the amino, thiol, carboxyl or aromatic groups of the desired amino acid residues of the polypeptide.

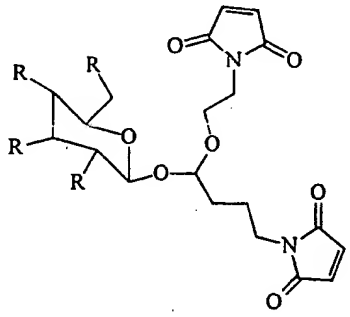
When the polypeptide is formed by peptide synthesis, non-encoded amino acids may be inserted with activatable side-chains at particular points in the sequence. Upon activation, these residues will either link to an agent or to another point in the polypeptide chain. One example is the amino acid analog *p*-benzoyl-L-phenylalanine, the synthesis of which is described by Cauer et al. (*J. Biol. Chem.*261:10695-10700, 1986.

If the activating enzyme is a nuclease, the cross-linking agent can comprise a double-stranded oligonucleotide that the enzyme is capable of cleaving. In a variation of this type of prodrug, the cross-linking agent comprises a single-stranded oligonucleotide sequence complementary to a target nucleotide sequence at the target site, and is activated by cleavage of the duplex between the cross-linking moiety and the target nucleotide by a nuclease in the milieu. The complementary sequence is usually between 4 and 100 nucleotides in length, and is more typically about 20 to 40 nucleotides in length. Exemplary target sequences include the nucleic acid sequences of infectious pathogens such as bacteria and viruses, including, for example, mycobacteria tuberculosis, streptococcus, N. gonorrhea, HIV, herpes viruses such as cytomegalovirus, Epstein Barr virus, varicella zoster virus and herpes simplex, hepatitis and chlamydia.

This invention also contemplates cascading reactions, where the local activating enzyme activates a proportion of the prodrug, and the activated drug has enzymatic activity specific for additional prodrug in the administered composition.

Certain cross-linkers of use for forming enzymatically activatable prodrugs have the formula $W(CH_2)_n$-X-CH(OY)-$(CH_2)_n$-Z wherein W and Z are each a functional group selected from the group consisting of maleimide, succinimide and thiocyanate; *n* is a number from 1 to 10; X is oxygen, sulfur or nitrogen; and Y is an enzymatically cleavable moiety selected from the group consisting of galactose, mannose, glucose, phosphate, butyrate and acetate.

A homobifunctional cross-linking agent comprising a cleavable glycosyl acetal moiety is shown below. Upon removal of the glycosyl residue by the action of an activating glycosidase enzyme, the hydroxy acetal is generated which spontaneously hydrolyses. The net result is cleavage within the cross-linking agent and consequent conversion of the prodrug into the active form. These glycosyl-containing cross-linking agents have the formula



wherein each R is independently hydroxy or acetate.

Design of cyclized prodrugs

The development of a cross-linked prodrugs from a prototype peptide drug involves the steps of: inserting the cross-linking agent into the prototype polypeptide; and, if the cross-linking agent is not itself cleavable by the activating enzyme, also inserting a cleavable heterologous sequence. (The term "inserting" refers to design of the prodrug structure, wherein the inserted component is not found in exactly the same context in nature; no implication is intended as to the method of manufacture of the prodrug). Any arrangement of cross-link and heterologous sequence that prevents reconstitution of the function of the activated form before cleavage, but permits activation after cleavage is suitable, and may be determined empirically.

The prodrugs of this invention are typically designed from a single proteinaceous effector molecule. The effector molecule may naturally occur in the active form as an assembly of homologous or heterologous peptide chains, or as a single chain form. The cross-linked prodrug may comprise a cross-linked form of a single chain or chain fragment of the naturally occurring counterpart, or a fusion of multiple chains or chain fragments in a single polypeptide. Where reassembling involves association with a second component in the reaction mixture, the second

component may be homologous or heterologous to the first, and where heterologous need not be cross-linked.

A systematic approach to obtaining cross-linked prodrugs is to base the location of the cross-link and heterologous sequence upon known structural features of the prototype. The functional elements of prototype enzyme can be mapped onto their structure by other techniques known in the art, such as photoreactive cross-linking, characterization using a panel of monoclonal antibodies, and mutation analysis.

Ideally, full structural information of the prototype peptide drug is known by way of its amino acid sequence, its crystal structure, and the location of the active site for effector function or receptor binding. Sequence and coordinate data for a number of proteins is available through the Protein Database of the U.S. National Institutes of Health. Advanced computer algorithms are available (e.g., at the University of Oregon) for manipulating 3-dimensional projections of proteins, determining interacting surfaces of subunits and the effects of making amino acid substitutions or insertions.

Once structural features are known, possible positions for the cross-link are designed that prevent functional activity of the peptide when in place. For example, the cross-link may be positioned so as to prevent access to a binding or catalytic site, or to prevent access of a co-factor. Alternatively, the cross-link is positioned so as to prevent various portions or subunits of the drug from interacting with each other. Particularly suitable are portions that are involved in an internal hydrophobic or other non-covalently bonded interface between subunits or regions of the molecule. Thus, the presence of the cross-link prevents the interface from forming until activation occurs.

Where the cross-linking moiety is not itself cleavable by the activating enzyme, a heterologous sequence is positioned within the polypeptide so as to permit the drug to resume its function. Most usually, an inserted heterologous proteolytic cleavage recognition site is incorporated into the peptide sequence internally, between the two reactive amino acid residues used for the cross-linking reaction.

In combination (or as an alternative) to these predictive modeling techniques, cross-linked prodrugs of this invention are identified or refined by empirical testing. An effector fragment of the drug is pared down to the minimum number of amino acids necessary to reconstitute active function or reassemble subunits. Where multiple subunits are present, this is often more convenient with the smaller of the subunits in the assembled complex, in part, because of the ease of chemical synthesis of shorter sequences. Once the functional core of the peptide is identified, reactive amino acids can be added onto one or both ends that permit cross-linking around or back into the core. Generally, in any of the cross-linked prodrugs of this invention, the developer will aim for an activity in the cross-linked drug of less than about 10% of the non-crosslinked equivalent, preferably less than about 1%.

Once an inactive cross-linked form of the drug polypeptide has been successfully designed and obtained, a heterologous protein recognition sequence is optionally incorporated into the structure. A heterologous protease recognition sequence, if present, is conveniently inserted between the terminal reactive amino acid and the core, which helps ensure that cleavage by the

activating enzyme will open the cyclized polypeptide in a way that permits it to resume activity. Short neutral linking peptide groups (polygly/ala or helix forming structures, or relatively shapeless sequences with a proportion of charged side chains) may be added between the reactive amino acid and the recognition site, or the recognition site and the functional core, to permit access of the activating enzyme to the site or facilitate reconstitution.

Proof of concept for the prodrugs of this invention is derived in part from cyclized reassembling enzyme components that reassemble only upon cleavage of an enzyme recognition site incorporated in the polypeptide or the cross-link moiety of the cyclized element. A prototype enzyme with complementary components is β-galactosidase. A number of β-Galactosidase N-terminal enzyme donor sequences are known in the art. See U.S. Patent No. 4,708,929; Langley and Zabin, *Biochemistry 15*:4866 (1976); Zabin, *Mol. and Cellular Biochem. 49*:84 (1982); Henderson, *Clin. Chemistry 32*:1637 (1986); Khanna, *Amer. Clin. Lab. 8*:14 (1989) and Coty, *J. Clin. Immunoassay 17*:144 (1994). Experiments showing that cyclized β-galactosidase enzyme donors reconstitute activity after cleavage by an activating enzyme are described below in the example section.

A number other of enzymes are known or predicted to have complementing subunits. An active cytochrome c has also been reconstituted from subunits from different organisms. Corradin et al., 1979, J. Exp. Med. 149: 436-47. Complementation of lactose permease mutants by other permease mutants has been described. Bibi et al., 1990, *Proc. Natl. Acad. Sci. USA* 87: 4325-9; Bibi et al., 1992, *Proc. Natl. Acad. Sci. USA* 89: 1524-8; Wrubel et al., 1990, *J. Bacteriol.* 172: 5374-81. SecA analogues have also been reconstituted from amino-terminal and carboxyl-terminal fragments of SecA. Kimura et al., 1991, *J. Biol. Chem.* 266: 6600-6. Two overlapping fragments of staphlococcal nuclease are able to complement each other. he complementing heterologous pair consists of a 42 amino acid polypeptide, designated nuclease $T\text{-}P_{6\text{-}48}$, and a 100 amino acid residue, designated nuclease $T\text{-}P_{49,50\text{-}149}$. See Tamiuchi et al., 1969, *J. Biol. Chem.* 244:3864; Taniuchi et al., 1977, *J. Biol. Chem.* 252: 125-40; and EP patent application No. 0,243,797. Thioredoxin has also been reconstituted from peptide fragments. Slaby et al., 1975, *J. Biol. Chem.* 250: 1340-7; Tasayco et al., 1995, *Proteins* 22: 41-4.

A further example is based on the prototype enzyme ribonuclease. Bovine pancreatic ribonuclease subjected to limited digestion by subtilisin produces two fragments, S-peptide (residues 1-20) and S-protein (residues 21-124) that can be separated and reconstituted to give the fully active complex ribonuclease S. U.S. Patent No. 5,106,950 describes polypeptide-labeled analyte analogs for use in immunoassays and capable of binding a polypeptide partner. The lysines of S-peptide can be cross-linked using an amino-reactive homobifunctional reagent. Alternatively, they can be thiolated with 2-iminothiolane and then cross-lined with a sulfhydryl-reactive crosslinking reagent. Another candidate enzyme component comprises a cysteine residue at either end of the S-peptide sequence, optionally through a short linker sequence, which are then cross-linked to tie the two ends

of the S-peptide together. Heterologous protease recognition sequences can optionally be inserted during synthesis between one of the cysteines and the S-peptide core.

In principle, the approach for generating a prodrug of this invention is no different for a prototype having an effect other than an enzymatic effect. The structural considerations are the same; the only difference is that the read-out assay for empirical testing will be in a different form. Thus, for example, to test the effectiveness of a toxic effector drug, preliminary testing can be performed with susceptible cultured cells. In a first screening step, successful cross-linked derivatives will have no more than about 10%, preferably no more than about 1%, and still more preferably no more than about 0.1% of the toxic activity of the prototype when tested on the cells. In a second screening step, the cross-linked derivatives will regain preferably at least about 5%, more preferably at least about 50% of the native activity upon pretreatment with the activating enzyme. In an optional third screening step, the ability of the candidate prodrug to be activated in situ can be determined by comparing the ability of the prodrug to affect cells expressing the activating enzyme in comparison with cells that do not. In a similar way, a prodrug based on a cytokine or growth factor can be tracked through these steps by standard assays for cellular activity known to be affected by the native factor. Where the prodrug is intended for use in vivo, testing will ultimately advance to a suitable animal disease or in vivo targeting model.

By way of illustration, prodrugs of this invention may be based on ribonucleases such as ribonuclease A, barnase, angiogenin, or the *pseudomonas* exotoxin-barnase chimera of Prior et al. (supra). Since Domain II is reportedly required for translocation of the chimera into the cytosol and the barnase is required for the cytotoxic effect, a cross-link introduced into either region would interfere with activity. One candidate site is the terminal residues which are amino acids 604-613 of *pseudomonas* exotoxin, which apparently must be accessible for cytosolic translocation.

Preparation of prodrugs

The assembly of cyclized prodrugs and the more general practice of the invention will employ, unless otherwise indicated, conventional techniques of molecular biology, microbiology, cell biology, biochemistry and immunology, which are within the skill of the art. Such techniques are explained fully in such standard publications as "Molecular Cloning: A Laboratory Manual", second edition (Sambrook et al., 1989); "Oligonucleotide Synthesis" (M.J. Gait, ed., 1984); "Animal Cell Culture" (R.I. Freshney, ed., 1987); "Methods in Enzymology" (Academic Press, Inc.); "Handbook of Experimental Immunology" (D.M. Weir & C.C. Blackwell, eds.); "Gene Transfer Vectors for Mammalian Cells" (J.M. Miller & M.P. Calos, eds., 1987); "Current Protocols in Molecular Biology" (F.M. Ausubel et al., eds., 1987); "PCR: The Polymerase Chain Reaction", (Mullis et al., eds., 1994); "Current Protocols in Immunology" (J.E. Coligan et al., eds., 1991).

General methods for peptide synthesis are described *inter alia* in Stewart, J.M. and Young, J.D., "Solid Phase Peptide Synthesis", 2nd Ed., Pierce Chemical Company: Rockford, IL, 1984;

Atherton, E. and Sheppard, R.C., "Solid Phase Peptide Synthesis: A Practical Approach", IRL Press: New York, 1989; Jones, J., "The Chemical Synthesis of Peptides" (International Series of Monographs on Chemistry, No. 23), Clarendon Press: Oxford, 1991; and Barany, G. and Merrifield, R.B., "Solid Phase Peptide Synthesis", Chapter 1 (pp. 1-284) of "The Peptides", Vol. 2, Academic Press: New York, 1979. Additional publications and supplies may be obtained from Calbiochem-Novabiochem, San Diego, CA.

General methods for preparing polynucleotide-peptide and peptide-peptide complexes and a spectrum of labeling strategies are described in Hermanson, G.T., "Bioconjugate Techniques", Academic Press: New York, 1996.

Therapeutic peptides can be isolated from natural sources and adapted for use in this invention by standard biochemical techniques. Alternatively, polypeptides below ~60 amino acids in length are conveniently prepared by chemical solid-phase synthesis. See, for example, U.S. Patent No. 4,493,795 and the scientific literature cited therein. Polypeptides of any length can be obtained by recombinant expression of the corresponding polynucleotide encoding region in a suitable prokaryotic or eukaryotic expression system.

Chimeric therapeutic peptides can be made employing recombinant DNA methodologies by genetic insertion of the gene encoding the desired recognition site into the gene encoding the enzyme sequence at a suitable endonuclease recognition site. Plasmid vectors containing enzyme encoding DNA sequences are well known in the art. Recombinant polymerase chain reaction cloning can be performed employing oligonucleotide primers containing the coding sequence of interest and suitable restriction enzyme cloning sites to construct peptides having an internal protease susceptible sequence. Alternatively, chemical synthesis of the polypeptide and a protease recognition site from amino acid starting material by sequential addition of amino acids or protected amino acids to a growing peptide chain can be employed.

The prepared peptide is then cross-linked using the agent of choice and the purified. An inert, or non-cleavable, cross-linking moiety (for example bis-maleimidohexane) is usually employed where the peptide sequence of the prodrug contains a protease recognition site. Otherwise, a cross-linking agent cleavable by a nuclease, glycosidase, phosphatase, amidase, esterase, or other enzyme or environmental agent may be used. Such cross-linking agents are described in more detail elsewhere in this disclosure..

The cross-linking typically involves reacting the polypeptide with a cross-linking agent under reaction conditions suitable to cause the cross-linking agent to covalently attach to two reactive amino acid residues of the polypeptide, and then isolating the cross-linked peptide from the reaction mixture. Heterobifunctional cross-linking agents are convenient in many instances for linking between two different polypeptide chains or for positioning at least one end of the cross-link within the polypeptide. Thiol-reactive cross-linkers are also suitable for exact positioning by reacting with cysteine residues produced selectively from internal disulfide bonds by mild reduction. Alternatively, thiol groups can be introduced into the polypeptide by reaction with commercially available reagents

such as 2-iminothiolane; or amino acids with reactive groups can be introduced into the polypeptide during synthesis.

Subsequently, the cross-linked peptide mixture is separated into fractions by techniques such as HPLC or adsorption chromatography, and assayed for the desired activity as described elsewhere in this disclosure, thereby removing unreacted, polymerized, and inappropriately cross-linked contaminants.

Pharmaceutical compositions and their use

An effective amount of a prodrug of this invention may be used for treating an individual for a condition that is amenable to the therapeutic effects of the activated form of the drug.

An "individual" or "subject" treated according the methods of this invention is a vertebrate, particularly a mammal (including farm animals, sport animals, and pets), and typically a human.

"Treatment" refers to clinical intervention in an attempt to alter the natural course of the individual being treated, and may be performed either for prophylaxis or during the course of clinical pathology. Desirable effects include preventing occurrence or recurrence of disease, alleviation of symptoms, diminishment of any direct or indirect pathological consequences of the disease, such as hyperresponsiveness, inflammation, or necrosis, preventing metastasis, lowering the rate of disease progression, amelioration or palliation of the disease state, and remission or improved prognosis. The "pathology" associated with a disease condition is anything that compromises the well-being, normal physiology, or quality of life of the affected individual.

Treatment is performed by administering an "effective amount" of a prodrug-containing composition of this invention. An effective amount is an amount sufficient to effect a beneficial or desired clinical result, and can be administered in one or more doses.

The modes of treatment contemplated in this invention are varied, depending on the specificity of the enzyme sequence and the pharmacological effect of the activated drug.

Pharmaceutical compositions of this invention can be prepared for administration to an individual in need thereof, particularly humans, in accordance with generally accepted procedures for the preparation of pharmaceutical compositions. General procedures for preparing pharmaceutical compositions are described in Remington's Pharmaceutical Sciences, E.W. Martin ed., Mack Publishing Co., PA. Liquid pharmaceutically administrable compositions can, for example, be prepared by dispersing a prodrug in a liquid excipient, such as water, saline, aqueous dextrose, or glycerol, optionally including preservatives such as enzyme inhibitors to prevent activation of the prodrug during storage. The composition may optionally also contain other medicinal agents, pharmaceutical agents, and carriers.

Compositions for injection are typically supplied as liquid solutions or suspensions, but may also be supplied frozen or lyophilized for dissolution or suspension in liquid prior to injection. Although

not required, pharmaceutical compositions are in some instances supplied in unit dosage form suitable for administration of a precise amount.

The route of administration of a pharmaceutical composition depends, inter alia; on the intended target site, clinical condition, and the nature of the condition being treated. Systemic or local intravenous administration, injection directly into an affected site, and administration to a mucosal surface are the most usual routes. Pulmonary administration by aerosol is conducted using a nebulizer device. Apparatus and methods for forming aerosols are described in Kirk-Othmer, "Encyclopedia of Chemical Technology", 4th Ed Vol. 1, Wiley NY, pp 670-685, 1991.

The size of the dose is selected taking into account the expected volume of distribution of the composition before reaching the intended site of action, and then providing sufficient inhibitor to meet or exceed the IC_{50} concentration as measured in an appropriate cell bioassay, typically at about 2-20 times IC_{50} concentration, depending on any anticipated side-effects.

Of course, treatment options for the prodrugs of this invention are not limited to administration in vivo. The prodrugs may be used, for example, to administer to cultured cells in a manner that permits a selective effect on certain cell types within the population.

The assessment of the clinical features and the design of an appropriate therapeutic regimen for an individual patient is ultimately the responsibility of the prescribing physician.

Further illustration of the development and use of reagents and assays according to this invention are provided in the Example section below. The examples are provided as a further guide to a practitioner of ordinary skill in the art, and are not meant to be limiting in any way.

EXAMPLES

Example 1: *Intramolecular cross-linking of ED28 via native cysteine residues*

ED28 is an enzyme donor polypeptide comprising 90 amino acids and containing two cysteine residues at amino acid positions 23 and 68. Positions 23 through 73 comprise the N-terminus of native β-galactosidase (using the convention of numbering the N-terminal Met residue "1"). The sequence of ED28 is also disclosed in U.S. Patent No. 4,708,929, which describes how to make it. It was intramolecularly linked by forming a disulfide bond between the two cysteine residues at amino acid positions 23 and 68.

ED28, 2.5 mg, was dissolved in 50 mM sodium phosphate buffer, pH 8.5, containing 30% acetonitrile (0.5 ml). The solution was applied to a prepacked SEPHADEX™ G25 high molecular weight purification column (NAP5, ® Pharmacia, Inc.) which had been previously equilibrated with 5 column volumes of 30 mM sodium phosphate, pH 8.5, containing 39% acetonitrile. The ED28 was eluted with 1 ml of the same buffer. This procedure ensured removal of any low molecular weight reducing agents, such as dithiothreitol, which would prevent disulfide bond formation. The resultant

solution was incubated with stirring for 12 hours, after which time the ED28 was about 95% converted to a disulfide-bonded molecule.

The cross-linked ED was purified by reverse-phase HPLC on a C4 RPLC column (Vydac Protein C4, 25 cm x 10 mm). The column was developed at a flow rate of 4 ml/minute. A 23 to 33% gradient was established over a 45 minute time period using concentrations beginning with weak eluent of 0.1% trifluoroacetic acid (TFA) in H_2O and ending with strong eluent of 0.1% TFA in acetonitrile. A sample of the purified, intramolecularly cross-linked material was treated with a 10 mM solution of the reducing agent DTT and reinjected onto the HPLC. As expected, the elution profile corresponded to the linearized material.

To confirm inhibition of the complementation activity of the cysteine-linked ED28 polypeptide, a CEDIA assay (® Microgenics Corp., Concord, CA) was performed using this material in the presence and in the absence of DTT. Solutions of cross-linked and linear ED28 (20 pmol) were prepared and incubated with enzyme acceptor EA22 (20 U/test), which comprises the complementing β-galactosidase fragment with a deletion of the amino acids from positions 13 to 40, and CPRG (2 mg/ml) in a buffered solution (dipotassium hydrogen phosphate, 210 mM; potassium dihydrogen phosphate, 150 mM; sodium chloride, 400 mM; EGTA, 10 mM; magnesium acetate, 2 mM; methionine, 10 mM; TWEEN 20, 0.05 %; PLURONIC™ 101 (® BASF Corporation), 0.001%; Dextran T40, 4%; bovine serum albumin, 0.1%; sodium azide, 10 mM; pH 6.95) at 37°C for 4 minutes. The rate in absorbance at 574 nm was measured per minute between 4 and 6 minutes. The results are shown in Table I below.

TABLE I

Enzyme Donor	*Reducing Agent*	*mAU/min @ 574 nm*
Cross-linked ED28	None	42
Cross-linked ED28	DTT	344.5
Linear ED28	None	346
Linear ED28	DTT	345.2

These results demonstrate that the complementation activity of the cysteine-linked ED28 polypeptide was 12% of the ED28 which had been linearized by chemical reduction with DTT. The presence or absence of DTT had no effect on fresh, reconstituted linear ED28.

Example 2: *Intramolecular cross-linking of ED28 with a homobifunctional, acid-labile cross-linking moiety*

The acid-labile, homobifunctional cross-linking agent 2,2 bis-maleimidoethoxypropane (BMEP), which can be made following the method of Srinivasvachar, *Biochemistry 28*:2501 (1989), was used to cross-link ED28. This cross-linking agent contains two maleimide groups, which react rapidly and specifically with sulfhydryl groups to form stable covalent bonds. The linkage between

the two maleimide groups contains a ketal moiety that renders the cross-linker acid-labile. This cross-linking agent was used to form an intramolecular cross-link between the two cysteine residues of ED28.

ED28, 1.0 mg, was dissolved in 50 mM sodium phosphate buffer, pH 7.0, containing 39% acetonitrile (0.5 ml) to remove any low molecular weight reducing agents such as DTT which would prevent cross-linking. The solution was applied to a prepacked SEPHADEX™ G25 column previously equilibrated with 5 column volumes of 30 mM sodium phosphate, pH 7.5, containing 30% acetonitrile. The ED28 was eluted with 1 ml of the same buffer. To the eluent was added 11 x 0.1 equivalent aliquots of BMEP in acetonitrile (5 µl total volume) over a 30 minute time period. The reaction mixture was then incubated for 1 hour at room temperature, after which time the starting material was completely converted to cross-linked product.

The BMEP cross-linked ED28 was purified by reverse-phase HPLC on a C4 RPLC column. The column was developed at a flow rate of 4 ml/minute. Using a strong eluent isochratic profile of 24.5%, the purified material was eluted using concentrations of weak eluent of 100 mM triethylammonium acetate (TEAA) in H_2O and strong eluent of acetonitrile. The purified material was lyophilized and stored at -80°C. A sample of the purified material was reconstituted in water and the pH adjusted to 2.0 with 0.1 M HCl and incubated for 3 minutes at room temperature. The pH was then adjusted to 6.8 employing a buffered solution (see Example 1) and the complementation activity with enzyme acceptor protein determined as described in Example 1. The results are shown in Table II below.

TABLE II

Enzyme Donor	*Treatment*	*mAU/min @ 574 nm*
Cross-linked ED28	None	22.5
Cross-linked ED28	pH 2.0 for 3 min	925
Linear ED28	None	930
Linear ED28	pH 2.0 for 3 min	927

These results demonstrate that the untreated BMEP-linked ED28 has 2.5% of the complementation activity of the acid treated BMEP-linked ED28. Thus, the cross-linked ED28 can be linearized by mild acid hydrolysis of the chemical cross-linking moiety. A control experiment employing linear ED28 demonstrates that the 3 minute acidic pretreatment had no effect on the complementation of linear ED28.

Example 3: *Intramolecular cross-linking of ED28 via a homobifunctional cross-linker and protease cleavage of the cross-linked ED28 with endoprotease Glu-C*

This example describes the construction and use of an enzyme donor polypeptide that is cross-linked using a moiety that is not cleavable under assay conditions. In this example, the

recognition sequence for a protease analyte of interest is not incorporated into the cross-linker but rather into the amino acid sequence of the enzyme donor or attached to its N- or C-terminus. This can be accomplished by recombinant DNA techniques or by solid phase peptide synthesis techniques, both of which are well known to those of skill in the art. The action of the protease cleaves the cross-linked enzyme donor at the protease recognition site, thereby linearizing the intramolecularly cross-linked peptide and enabling its complementation with enzyme acceptor.

Demonstrating this concept, ED28 was cross-linked with the homobifunctional cross-linking agent bis-maleimidohexane. Use of this reagent results in the irreversible cross-linking of sulfhydryl moieties under mild conditions. See Partis, *J. Prot. Chem.* 2:263-77 (1983). ED28, 1.0 mg, was dissolved in 50 mM sodium phosphate buffer, pH 7.0, containing 30% acetonitrile (0.5 ml). The solution was applied to a prepacked SEPHADEX™ G25 previously equilibrated with 5 column volumes of 30 mM sodium phosphate, pH 7.0, containing 30% acetonitrile. The ED28 was eluted with 1 ml of the same buffer. To the eluent was added 11 x 0.1 equivalent aliquots of BMH in acetonitrile (5 µl total volume) over a 30 minute time period. The reaction mixture was then incubated for 2 hours at room temperature, after which time the starting material was completely converted to cross-linked product.

The BMH cross-linked ED28 was purified by reverse-phase HPLC on a C4 RPLC column. The column was developed at a flow rate of 4 ml/minute. Using a strong eluent isochratic profile of 28.5%, the purified material was eluted using concentrations of weak eluent of 0.1% trifluoroacetic acid (TFA) in H_2O and strong eluent of 0.1% TFA in acetonitrile. The purified material was lyophilized and reconstituted in 25 mM ammonium carbonate buffer, pH 7.8. The buffered sample was then incubated with 10 µg endoproteinase Glu-C protease (from *S. aureus* V8, Boehringer Mannheim) for 1 hour at room temperature. Enzyme acceptor protein (50 µl, 500 U/ml) and CPRG solution (50 µl, 3 mg/ml) were added, the plate was incubated at 37°C and the absorbance at 570 nm was measured. The results are shown in Table III below.

TABLE III

Sample	*mAU/min @ 570 nm*
Without protease treatment	25
With protease treatment	713

This Glu-C protease specifically cleaves peptides at the C-terminal side of glutamic acid residues. Hence, the specificity of this protease for glutamic acid residues 62 and 63 of ED28 was exploited to linearize the BMH cross-linked ED28 at those positions rather than via the BMH moiety. Upon treatment of the cross-linked enzyme donor polypeptide with Glu-C protease, activity was increased dramatically, about 24-fold, indicating that cleavage had occurred.

Example 4: *Preparation of homobifunctional bis-maleimidoacetal cross-linking agent*

Synthesis of N-(2-trimethylsiloxyethyl)-maleimide

As shown in **Figure 1,** to a solution of ethanolamine (1.8 g, 29.5 mmol) in saturated sodium bicarbonate solution (100 ml) was added N-methoxycarbonylmaleimide (95 g, 32.3 mmol) in portions with vigorous stirring at 0°C. The mixture was allowed to warm to room temperature and stirred for 1 hour. The pH of the mixture was adjusted to 6-7 by careful addition of concentrated sulfuric acid (5 ml). The resultant solution was freeze dried and the solid residue extracted with ethyl acetate (2 x 400 ml) by stirring for 30 min. with each extraction. The ethyl acetate extracts were collected by filtration and evaporated in vacuo to afford N-(2-hydroxyethyl)maleimide (formula **I**) as a white solid (4.0 g, 96% yield); thin layer chromatography (TLC), Rf = 0.27, ethyl acetate/petroleum ether 1:1.

To a solution of N-(2-hydroxyethyl)maleimide (0.2 g, 1.41 mmol) in dry dichloromethane (10 ml) and triethylamine (0.22 ml, 1.57 mmol) at 0°C was added chlorotrimethylsilane (TMS-Cl, 0.2 ml, 1.57 mmol). After stirring for 1 hour at room temperature, TLC analysis (ethyl acetate/petroleum ether 1:1) indicated one spot (Rf = 0.67). Solvent was removed in vacuo and the residue was dissolved in dichloromethane and filtered through a small silica gel column eluting with dichloromethane. The fractions containing product were pooled and evaporated in vacuo to afford N-(2-trimethylsiloxyethyl)-maleimide (formula **II**) as colorless flakes (0.3 g; 100% yield).

Synthesis of 4-maleimidobutyraldehyde

As shown in **Figure 2,** to a solution of 4-aminobutyraldehyde diethylacetal (5 g, 31 mmol) in 100 ml of saturated bicarbonate was added N-methoxycarbonylmaleimide (4.91 g, 31.6 mmol) at 0°C (icebath). After 15 minutes, tetrahydrofuran (100 ml) was added at room temperature and the resultant mixture stirred for 1 hour. The resultant mixture was then acidified with 1 N hydrochloric acid to pH 6-7 and extracted with ethyl acetate (3 x 200 ml). The combined organic extracts were dried ($MgSO_4$) and evaporated in vacuo. The crude product was purified by chromatography on silica gel 60 eluting with ethyl acetate/petroleum ether 1:2 to yield 4-maleimidobutyraldehyde diethylacetal as a yellow oil (4.31 g; 58%), TLC Rf = 0.65, ethyl acetate/hexane 1:1.

4-Maleimidobutyraldehyde diethylacetal (2 g, 8.29 mmol) in tetrahydrofuran (20 ml) and water (0.5 ml) was stirred under argon and DOWEX™ 50X8 ion exchange resin (H^+, 2 g, ®Dow Chemical Co.) was added. After 12 hours stirring at room temperature, the solvent was decanted, dried ($MgSO_4$) and evaporated in vacuo to afford 4-maleimidobutyraldehyde (formula **III**) as a yellow oil which rapidly solidified on standing (1.38 g, 99%); TLC Rf = 0.4, ethyl acetate/hexane 1:1. The 4-maleimidobutyraldehyde turned out to be extremely unstable, so that it was necessary to do all work-up at low temperatures (0°C) and under complete exclusion of oxygen (inert atmosphere).

Synthesis of 1,7-bismaleimido-4-O-(tetraacetyl-β-D-galactopyranosyl)-5-oxaheptane

As shown in **Figure 3**, to a stirred solution of 4-maleimidobutyraldehyde (1.82 g, 11 mmol), N-(2-trimethylsiloxyethyl)-maleimide (0.79 g, 3.5 mmol), 1-trimethylsilyloxy-2,3,4,6-tetra-O-acetyl-β-

D-galactopyranose (1.52 g, 3.5 mmol) and molecular sieve [4 Å] in 40 ml dry dichloromethane was added TMSOTf (trimethylsilyl triflate, 0.67 ml, 3.5 mmol) at -78°C under rigorous dry conditions and inert atmosphere (argon). The reaction mixture was quenched by addition of 1.5 ml triethylamine/methanol (1:1) after two days. The solvent was removed in vacuo after CELITE (® Celite Corp.) filtration (5 g). The crude product was chromatographed (ethylacetate/light petroleum, 1:1) to give 1,7-bismaleimido-4-O-(tetraacetyl-β-D-galactopyranosyl)-5-oxaheptane (formula **IV**) (1.78 g, 2.7 mmol) in 77% yield.

Deprotection of 1,7-bismaleimido-4-O-(tetraacetyl-β-D-galactopyranosyl)-5-oxaheptane

To a stirred solution of 1,7-bismaleimido-4-O-(tetraacetyl-β-D-galactopyranosyl)-5-oxaheptane (200 mg, 0.3 mmol) in 20 ml dry methanol was added $Zn(OAc)_2$ (60 mg, 0.3 mmol). The solution was refluxed for 9 hours under rigorous dry conditions. Tert-butylmethylether (20 ml) was added at room temperature. CELITE filtration (5 g) with 200 ml tert-butylmethylether/methanol (1:1), evaporation of solvent in vacuo, followed by chromatography (ethylacetate/methanol/triethylamine, 4:1:1) gave a mixture of 1,7-bismaleimido-4-O-(β-D-galactopyranosyl)-5-oxaheptane (formula **IV**) and 1,7-bis-(3'-methoxysuccinimido)-4-O-(β-D-galactopyranosyl)-5-oxaheptane (formula **V**) in a ratio of 1:2 (145 mg, 0.29 mmol) in 99% yield. $Zn(OAc)_2$ was dried at 80°C (p=0.001 Torr) for 24 hours.

The 1,7-bismaleimido-4-O-(tetraacetyl-β-D-galactopyranosyl)-5-oxaheptane was used to prepare cyclic fusion peptides comprised of β-galactosidase enzyme donor and HIV gag sequences, constructed as set forth in Example 5 below.

Example 5: *Preparation of double Cys- HIV peptide-containing enzyme donors*

Double cysteine-containing enzyme donor polypeptides which also contained an HIV protease recognition site were prepared by recombinant DNA techniques and by solid phase peptide synthesis.

E. Coli strain AMA1004 (Casadaban, *Methods in Enzymology 100*:293, 1983) was used for expression of EA, ED, and complemented β-galactosidase. E. coli strain MC1061 (Meissner, *Proc Nat Acad Sci 84*:4171, 1987) was used for isolation of recombinant clones.

Oligonucleotide primers were designed to amplify the β-galactosidase alpha region known as ED7 from the plasmid pI87 with the addition of either the 8 residue (p17/p24) or 10 residue (p6/PR) HIV protease recognition sites carrying a HindIII restriction site for clone selection. The N-terminal primer for amplification of the ED7-HIV pI7/p24 gene (5'-GATACGAATTCTCAGAACTATCCGATCGTTCAGTCACTGGCCGTCGTTTTACAA-3') (SEQ ID NO:6) contained the 8 residue HIV protease recognition site.

The N-terminal primer for amplification of the ED7-HIV p6/PR gene (5'GATACGAATTCTGTAAGCTTTAACTTTCCGCAGATCACCCTGCTGGCCGTCGTTTTACAA-3') (SEQ ID NO:7) contained the 10 residue HIV protease recognition site. Both amplifications used the C-terminal primer KM1 (5'-CTGGCTTAACTATGCGGCATC-3') (SEQ ID NO:8). PCR amplifications

were run in an MJ Research minicycler PTC-150 beginning with denaturation at 94°C for 1 minute followed by 40 cycles of 92°C for 40 seconds, 65°C for 40 seconds, and 75°C for 1.5 minutes and a final elongation step of 75°C for 5 minutes. Reactions were 100 µl volumes and run as hot starts using PCR Gems (Perkin/Elmer).

Amplified DNA was cleaned by phenol-chloroform extractions and precipitated in ethanol. Resuspended material was trimmed by EcoR1 and Sal1 digestion and purified by agarose gel electrophoresis. Gel purified insert DNA was ligated into pl87 EcoR1/Sal vector. The resulting clone carried the ED7 gene with either the HIV pl7/p24 or p6/PR cleavage site as an internal gene fusion cassette inserted at an EcoR1 site located near the 3' end of the ED7 gene. The correct clone was identified by the presence of the HindIII site located in the PCR product and verified by DNA sequencing. For expression and purification, the ED7-HIV genes were transferred by BamH1/Sal1 digestion into a BamH1/Sal1 vector (p43) carrying the large fragment of β-galactosidase, EA46, which complements in vivo with the ED7-HIV gene products.

ED7-HIV pl7/24, ED7-HIV p6/PR and EA46 proteins were induced at 40°C from the lambda PL promotor through inactivation of the plasmid CI857 repressor. The cells were harvested after 4 hours of induction, and the complemented β-galactosidase was purified by a 40% ammonium sulfate precipitation followed by ion exchange chromatography on Q-SEPHAROSE™. The complemented enzyme was denatured in 10 M urea, and the recombinant ED-HIV proteins were separated from denatured EA46 by size exclusion chromatography in 6 M urea. Fractions containing the ED-HIV proteins were concentrated with an Amicon stir cell and dialyzed into a neutral TRIS buffer. Any residual contaminating proteins were removed through ion exchange chromatography on Q-SEPHAROSE.

The target peptides were also synthesized on an Applied Biosystems (ABI) Model 431A solid phase peptide synthesizer, using Fmoc protected amino acids activated with 2-1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU; ABI user bulletin #33). The synthesis was carried out at 0.25 mmol scale, and a preloaded HMP resin was used as the solid phase. The deprotection and coupling times were extended from the standard times recommended by the manufacturer. The following amino acids were used: Fmoc-Ala, Fmoc-Arg (Pmc), Fmoc-Asn (Trt), Fmoc-Asp (OtBu), Fmoc-Cys (Trt), Fmoc-Gln (Trt), Fmoc-Glu (OtBu), Fmoc-Gly, Fmoc-His (Trt), Fmoc-Leu, Fmoc-Lys (Boc), Fmoc-Phe, Fmoc-Pro, and Fmoc-Ser (tBu). The N-terminus was not acetylated and the C-terminus was left as the carboxy form.

Cleavage of the crude peptide-resin was accomplished by incubation for 3 hours in a solution of TFA containing the carbonium scavengers, water (4%), thioanisole (4%), phenol (1.5%), and 1,2-ethane dithiol (2%). The mixture was filtered, evaporated to an oil and precipitated with cold diethyl ether. Purification of the crude peptide was done by reverse-phase HPLC, using a Vydac 2.2 x 300 mm C18 column and a 16-41% acetonitrile/water gradient, with 0.1% TFA as the counter-ion. The purified peptide was designated SED35 and contained the VSFNFPQITL (SEQ ID NO:2) protease cleavage site.

Example 6: *Cross-linking ED7-HIV fusion peptide with 1,7-bismaleimido-4-O-(tetraacetyl-β-D-galactopyranosyl)-5-oxaheptane*

The ED7-HIV fusion peptide from Example 5 was then cross-linked by the covalent linkage of 1,7-bismaleimido-4-O-(tetraacetyl-β-D-galactopyranosyl)-5-oxaheptane between residues 10 and 53 of the fusion peptide.

The ED7-HIV fusion peptide ED7-HIV pI7/p24 was reconstituted in 50 mM sodium phosphate buffer, pH 7.0(1 ml), and the solution was applied to a prepacked SEPHADEX™ G25 column. The fusion peptide was eluted with the same buffer (1.5 ml). To the eluent containing 362 μg/ml of the fusion peptide (97% recovery) was added 11 x 0.1 equivalent aliquots of 1,7-basmaleimido-4-O-(tetraacetyl-β-D-galactopyranosyl)-5-oxaheptane in acetonitrile (5 μl total volume; 38 μg total) over a 30 minute time period. The reaction mixture was then incubated for 30 minutes at room temperature.

The cross-linked ED-HIV fusion peptide was purified by HPLC employing a Vydac Protein C4 (25 cm x 10 mm) semi-preparative HPLC column using 100 mM TEAA, pH 6.5 as solvent A and acetonitrile as solvent B. The column was developed at a flow rate of 4 ml/minute. A 22 to 40% gradient of solvent B was established over a 15 minute time period. Linear ED-HIV fusion peptide exhibited a retention time of 13 minutes. Cross-linked ED-HIV fusion peptide exhibited a retention time of 14.2 minutes. Fractions containing the product were pooled and lyophilized. The yield of cross-linked ED-HIV fusion peptide was 155 micrograms.

To test for complementation activity, samples of the linear ED-HIV fusion peptide and the cross-linked ED-HIV fusion peptide were reconstituted in assay buffer and serially diluted across a microliter plate. Enzyme acceptor (2050 U/ml) and CPRG (1 mg/ml), 50 μl each, were added to each well and the change in absorbance at 570 nm monitored every 30 seconds in a V max plate reader. The results are shown in Table IV below:

TABLE IV

Enzyme Donor	*Concentration*	*mAU/min @ 570 nm*
Linear ED-HIV fusion peptide	1.84 pmols	57.25
Cyclic ED-HIV fusion peptide	1.84 pmols	1.29
Linear ED-HIV fusion peptide	3.68 pmols	85.18
Cyclic ED-HIV fusion peptide	3.68 pmols	2.17

These results demonstrate that the complementation activity of the cross-linked enzyme donor was 2.5% that of the linear enzyme donor. A second HPLC purification was carried out using the once-purified material and the repurified cyclic ED-HIV fusion peptide tested again in this assay. In this test, the cyclic ED-HIV fusion peptide exhibited only 0.04% of the complementation activity

exhibited by the linear ED-HIV fusion peptide, indicating that the higher activity seen after only one HPLC purification is likely due to the presence of linear ED-HIV contaminant.

Example 7: *Cross-linking SED35-HIV fusion peptide with 1,7-bismaleimido-4-O-(tetraacetyl-β-D-galactopyranosyl)-5-oxaheptane*

SED35 is a 60 residue fusion peptide produced by solid phase peptide synthesis that contains a 14 residue N-terminal sequence composed of the decapeptide VSFNFPQITL (SEQ ID NO:2) corresponding to the p6/PR HIV-1 protease cleavage site and amino terminal residues GGGC in the HIV gag/pol polypeptide and a 46 residue C-terminal sequence composed of residues 28 through 73 of ED28.

SED35, (0.5 mg, 75 nmols) in 100 mM phosphate buffer, pH 6.5, containing 5 mM EDTA and 30% acetonitrile was combined with 5 µl aliquots of 6 x 0.2 mol equivalents of 1,7-bismaleimido-4-O-(tetraacetyl-β-D-galactopyranosyl)-5-oxaheptane in acetonitrile (57 µg total) over a 30 minute period of time. The reaction mixture was then allowed to incubate 30 minutes at room temperature.

The cross-linked ED-HIV fusion peptide was purified by HPLC employing a Vydac Protein C4 (25 cm x 10 mm) semi-preparative HPLC column using 100 mM TEAA, pH 6.5 as solvent A and acetonitrile as solvent B. The column was developed at a flow rate of 4 ml/minute. A 35 to 40% gradient of solvent B was established over a 20 minute time period. Fractions containing the cross-linked ED-HIV fusion peptide were pooled.

Example 8: *ELISA assay format for determining HIV-1 protease inhibitor*

An ELISA plate format was used to determine IC_{50} values (concentration of inhibitor that causes a 50% reduction in enzyme activity) of various inhibitors using a kinetic microliter plate reader.

A stock solution of the cross-linked chimeric peptide SED35-HIV p6/PR containing HIV-1 substrate sequence VSFNFPQITL (SEQ ID NO:2) corresponding to the p6/PR cleavage site of the HIV gag/pol polypeptide was prepared at a concentration of 1 µg/ml in protease assay buffer (100 mM NaOAc, 1 M NaCl, 0.1% BSA, 1 mM EDTA, pH 5.0). Recombinant HIV protease was prepared at a concentration of 10 µg/ml, also in protease assay buffer. HIV protease inhibitors were dissolved in dimethyl sulfoxide (DMSO) and diluted to working concentrations in protease assay buffer containing 10% DMSO. β-Galactosidase enzyme acceptor protein EA22 was prepared in β-galactosidase assay buffer to give a final concentration of 500 U/ml. CPRG was dissolved in β-galactosidase assay buffer at a concentration of 3 mg/ml.

HIV protease inhibitors 94-001, 94-002, 94-003, 94-004 and 94-005 were dissolved in DMSO to give stock concentrations of 394 nM, 78.8 nM and 15.76 nM. See Ghosh et. al., *J. Med. Chem.* 37:1177-88 (1994) and Ghosh et. al., *J. Med. Chem.* 37:2506–8 (1994). Recombinant HIV-1 protease (homodimer, Mr = 22,000) stock solution (10 µl, 4.54 pmols), protease inhibitor stock

solutions (10 µl), cyclic ED-HIV stock solution (25 µl, 3.57 pmols), EA22 solution (50 µl, 25 U) and CPRG solution (50 µl) were pipetted into the wells of a polystyrene microtiter plate. The plate was incubated for 5 min. at 37°C and then the absorbance at 570 nm monitored for 20 min. The IC_{50} value for each of the inhibitors was determined from graphs of Vmax vs time. (The IC_{50} is proportional to the Ki value for the inhibitor). The relative IC_{50} values found for each of the inhibitors is given in Table V.

TABLE V

Inhibitor	*Relative IC_{50} (nM)*
94-001	5
94-002	4
94-003	2.5
94-004	3
94-005	7.5

Example 9: *COBAS MIRA assay format for determining HIV-1 protease inhibitor*

A three reagent assay system was used to determine IC_{50} concentrations of various inhibitors. A COBAS MIRA analyzer (® Roche Diagnostic Systems, Inc., Nutley, NJ) was used.

Sample (inhibitor 94-001, 94-002, 94-003 or 94-004) was diluted with an HIV protease buffer (10 mM sodium acetate, 1 M NaCl, 1 mM EDTA, 0.1% BSA, pH 5.0) modified with 10% DMSO to give final reagent inhibitor concentrations of 45.3 mM to 4.53 nM by dilution factors of ten.

Reagent 1 (R1) contained HIV protease diluted to a reagent concentration of 45 nM in HIV protease buffer.

Reagent 2 (R2) contained cross-linked enzyme donor SED35 at 0.30 mM and CPRG at 43 mg/ml in HIV protease buffer.

Reagent 3 (R3) contained EA22 diluted to a reagent concentration of 1315 U/ml.

The COBAS MIRA apparatus was programmed to deliver 10 µl of sample and 100 ml of R1 at time point one (T=0 minutes), 10 ml of R2 at time point two (T=2 minutes), and 95 ml of R3 at time point three (T=7 minutes). Rate values (absorbance changes within various time periods) were taken at time point four (T=9 to 11 minutes). These values were used to construct rate versus the log of inhibitor concentration graphs to determine IC_{50} values. All assays were performed at 37°C. The data given in Table VI for inhibitor 94-001 is exemplary.

TABLE VI

Inhibitor Concentration (M)	*Rate (mAU/min)*
2.1×10^{-7}	849
2.1×10^{-5}	247
2.1×10^{-3}	90

Using this data, a rate versus log inhibitor concentration graph was constructed and a curve fitting program was applied to generate a logarithmic line equation, in this case f(x) = -169*1n(x)+602, or f(y) = -9.19*1n(y)+60.9. The midpoint of the rate data (50% response) was then determined and this value inserted into the f(y) equation to generate the IC_{50} concentration. Here, the midpoint value was 470 mAU/min and the IC_{50} value was 4.35 nM. The results for the assay are given in Table VII.

TABLE VII

Inhibitor #	*IC_{50} (nM)*
reference	9.00
1	4.35
2	5.11
3	4.30
4	4.41

All publications and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference. The invention now being fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit or scope of the appended claims.

Example 10: *Cross-linked enzyme components with alternative protease recognition sequences*

Alternative enzyme components were prepared in which the HIV protease recognition sequence was substituted with the recognition sequence for another protease. The recognition sequence is conveniently added to the N-terminal side of the core enzyme polypeptide sequence, since this is near the last few cycles of peptide synthesis. The sequences are then capped through a polyglycine linker to a second cysteine that permits cross-linking via a thio-reactive cross-linking agent.

Figure 4 provides sequence listings and related data for three such enzyme components that have been prepared. The first enzyme component (SEQ. ID NO:9) contains the ICE protease recognition sequence YVAD (SEQ. ID NO:10) linked onto the β-galactosidase enzyme donor core.

The second enzyme component (SEQ. ID NO:12) contains the Caspase recognition sequence DEVD (SEQ. ID NO:13) linked onto the enzyme donor core. The third enzyme component (SEQ. ID NO:15) contains the IgA protease recognition sequence TPPTPSPS (SEQ. ID NO: 16), linked onto the enzyme donor core.

In trial reactions, the enzyme component with recognition site for ICE protease was crosslinked using bis-N-maleimido-1,6-hexane (BMH) or N,N'-bis(3-maleimidopropionyl)-2-hydroxy-1,3-propanediamine (BMP); homobifunctional cross-linking agents with specificity for sulfhydryl groups. The reaction mixtures were fractionated by HPLC using a gradient of acetonitrile to remove unreacted component and components that were dimerized via inter-chain linkage. Fractions were reconstituted in 250 µL 0.1% TFA in H_2O.

The fractions were then tested in enzyme complementation assays, in which the ICE protease, the complementing enzyme component (β-galactosidase enzyme "acceptor"), and the chromogenic β-galactosidase substrate CPRG were supplied.

In one experiment, the assay system was tested for the effect of the buffer on the reaction. CEDIA® type enzyme immunoassays are typically performed in a standard buffer containing potassium phosphate (pH 6.9), NaCl, EGTA, magnesium acetate, sodium azide, and the detergent TWEEN™ 20. ICE protease reactions have been described as optimally performed in a buffer of 100 mM HEPES (pH 7.5), 10% sucrose, 0.1% CHAPS™ detergent, and 10 mM dithiothreitol (DTT). Results for fractions "W11" and "W13" are shown below.

TABLE VIII

Buffer	*Fraction*	*Absorbance 570-650 nm*
reference		-0.1
ICE	W11	296
CEDIA	W11	85.1
ICE	W13	4.0
CEDIA	W13	25.2

With the W11 fraction, the ICE buffer was found to support somewhat better β-galactosidase activity than the standard CEDIA buffer.

In another experiment, various cross-linked enzyme components were compared. 10 µL of a crude ICE protease preparation was combined with 20 µL ICE buffer and 5 µL enzyme component, and incubated for 10 min at 37°C. After the first incubation, 55 µL CEDIA buffer, 50 µL of the enzyme acceptor EA22, and 50 µL of CPRG (2 mg/mL) were added, and the reaction mixture was incubated a further 20 min at room temperature. Absorbance was measured at 570-650 nm. Results are shown in Table IX:

TABLE IX

ICE protease dilution	*Fraction W11 (uncrosslinked)*	*BMH Fraction 4*	*BMD Fraction 6*
0	85.1	80.4	232
Neat	62.6	348	473
1:10	67.4	225	336
1:100	18.2	105	237
1:1000	42.7	65.6	229
No enzyme donor	0.0	0	0

The results show that amongst the fractions tested, BMH Fraction 4 had the best signal:noise ratio (i.e., difference in activity in the absence and presence of ICE protease).

In another experiment, the incubation of ICE protease with the enzyme donor was extended to 30 min before the acceptor and substrate were added, and several of the BMH fractions were compared with each other. A kinetic read was performed at 570-650 nm.

Results are shown in Figure 5. The upper panel is a chart of absorption data for BMH fractions 4, 5, 6, and 7, compared with fraction W11. Good signal:noise ratios were observed for both fractions 4 and 5. The lower panel shows the time-course of the β-galactosidase product observed for BMH cross-linked enzyme donor fraction 5 preincubated in the absence (open symbols) or presence (closed symbols) of ICE protease. The reaction reaches a constant velocity within a few minutes and is dependent on the presence of ICE protease in the original assay mixture.

CLAIMS

What is claimed as the invention is:

1. A cross-linked prodrug, having an inserted enzyme recognition site in a polypeptide of the prodrug and at least one covalent intrachain cross-link between amino acid side chains of the polypeptide, wherein the cross-linked prodrug is converted to a therapeutically more effective form upon cleavage of the enzyme recognition site.

2. The cross-linked prodrug of claim 1, which is converted to a therapeutically more effective form upon cleavage of the enzyme recognition site, followed by combination of the cleaved prodrug with one or more additional polypeptides to create the more effective form.

3. The cross-linked prodrug of claim 1 or claim 2, wherein the enzyme recognition site is cleavable by a viral or bacterial protease.

4. The cross-linked prodrug of claim 1 or claim 2, wherein the enzyme recognition site is cleavable by a protease that is expressed at elevated levels at sites of inflammation or malignancy.

5. The cross-linked prodrug of claim 1 or claim 2, wherein the enzyme recognition site is cleavable by a protease selected from the group consisting of HIV protease, N. gonorrhea protease, Glu-C protease, ICE protease, Caspase, cathepsin D, PSA, and IgA protease.

6. The cross-linked prodrug of any preceding claim, wherein the intrachain cross-link is a disulfide bond between cysteine residues or thiolated amino groups.

7. The cross-linked prodrug of any preceding claim, wherein the intrachain cross-link is formed by contacting the polypeptide with a homo- or heterobifunctional cross-linking agent.

8. A cross-linked prodrug, having an inserted enzyme recognition site in a polypeptide of the prodrug and at least one cross-linking moiety covalently bonded between amino acid side chains of the polypeptide, wherein the cross-linking moiety comprises an enzymatically cleavable site, and wherein the cross-linked prodrug is converted to a therapeutically more effective form upon cleavage of the enzymatically cleavable site.

9. The cross-linked prodrug of claim 8, which is converted to a therapeutically more effective form upon cleavage of the enzyme recognition site, followed by combination of the cleaved prodrug with one or more additional polypeptides to create the more effective form.

10. The cross-linked prodrug of claim 8 or claim 9, wherein the cleavable site is cleavable by an endoglycosidase.

11. The cross-linked prodrug of claim 8 or claim 9, formed by contacting the polypeptide with a cross-linking agent of the formula $W\text{-}(CH_2)_n\text{-}X\text{-}CH(OY)\text{-}(CH_2)_n\text{-}Z$ wherein:

W and Z are a nucleophilic leaving group;

n is a number from 1 to 10;

X is oxygen, sulfur or nitrogen; and

Y is an enzymatically cleavable moiety selected from the group consisting of glycosides, acetylated glycosides, phosphate, butyrate and acetate.

12. The cross-linked prodrug of claim 8 or claim 9, wherein the cross-linking moiety comprises a polynucleotide sequence with a recognition site for a nuclease.

13. The cross-linked prodrug of any of claims 1 to 12, wherein the therapeutically more effective form is at least 10 times more effective than the prodrug.

14. The cross-linked prodrug of any of claims 1 to 12, wherein the therapeutically more effective form is a toxin.

15. The cross-linked prodrug of any of claims 1 to 12, wherein the therapeutically more effective form is a cytotoxin or an immunotoxin.

16. The cross-linked prodrug of any of claims 1 to 12, wherein the therapeutically more effective form comprises cytosolic translocation sequence or RNase activity.

17. A method of preparing the prodrug of any of claims 1 to 16, comprising cyclizing a cytotoxin or an immunotoxin by cross-linking two amino acids in the polypeptide.

18. A method of treating a subject for a disease associated with increased local expression of an enzyme, comprising administering to the subject a cross-linked prodrug according to any of claims 1 to 16, wherein the locally expressed enzyme is specific for the enzyme recognition site or the enzyme cleavable site of the prodrug.

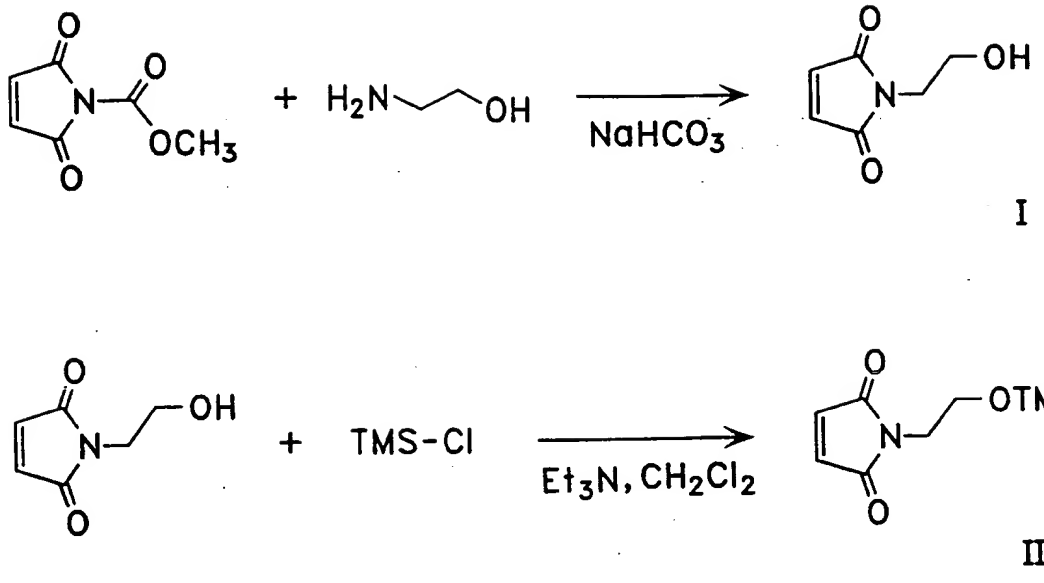

+ H_2N OH
$NaHCO_3$
OH
I
OH
+ TMS-Cl
Et_3N, CH_2Cl_2
OTMS
II

Fig. 1

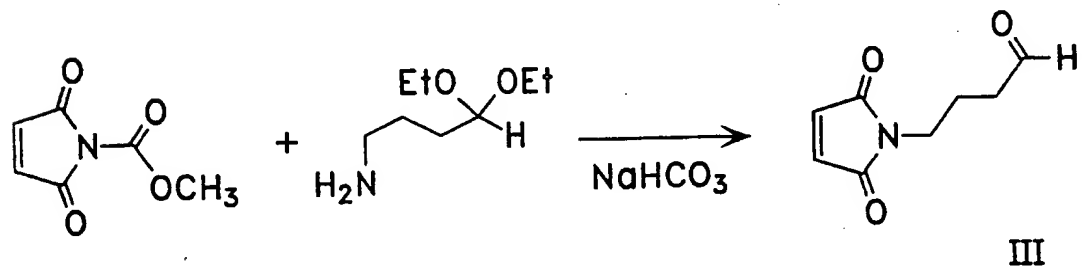
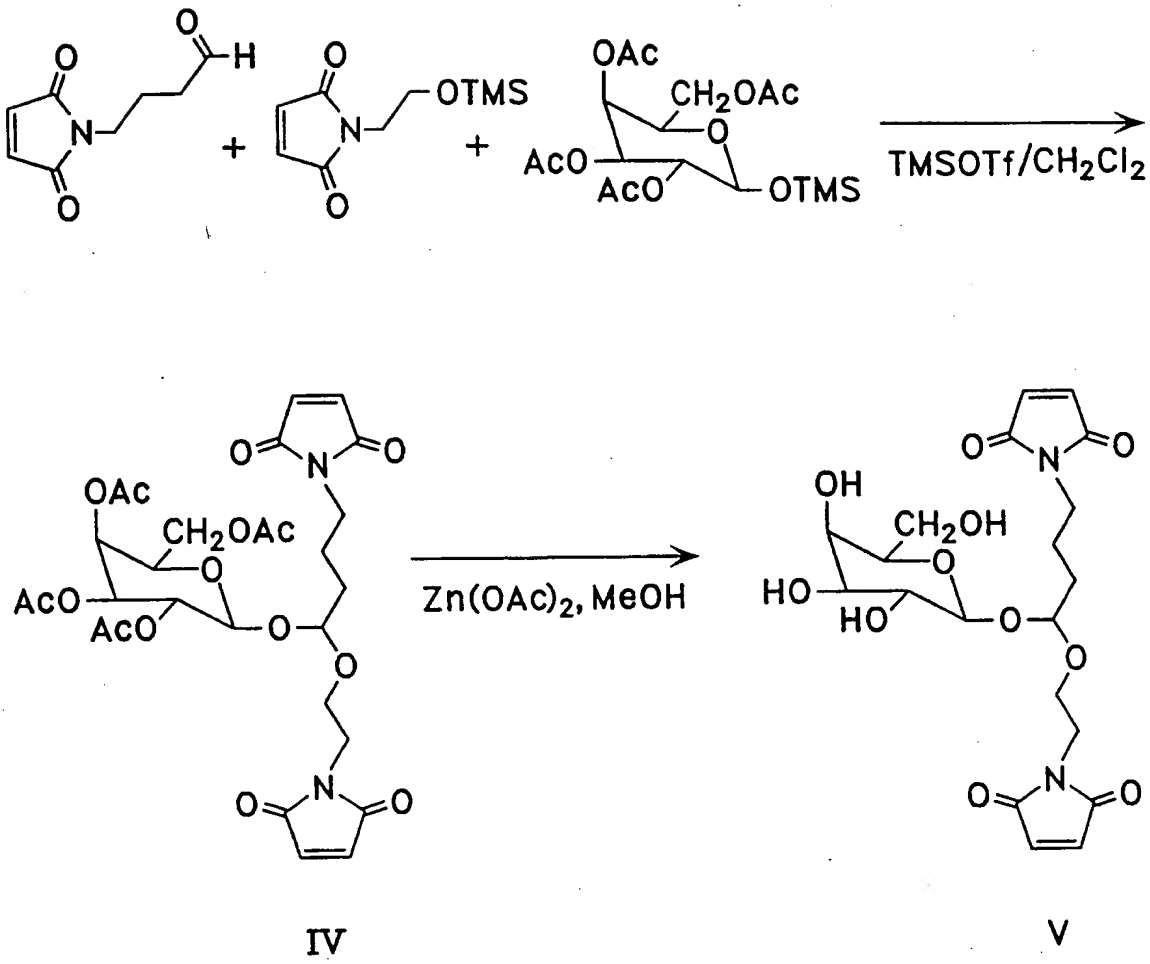

OCH_3
+
EtO OEt
H
H_2N
$NaHCO_3$
H
III

Fig. 2


OTMS
OAc
CH2OAc
AcO
AcO
OTMS
TMSOTf/CH2Cl2
Zn(OAc)2, MeOH
OH
CH2OH
HO
HO

IV V

Fig. 3

Chemistry : Fmoc
Composition:
Sequence : [H]-Cys-Gly-Gly-Gly-Tyr-Val-Ala-Asp-Gly-Ser-Leu-Ala-Val-Val-Leu-Gln-Arg-Arg-Asp-Trp-Glu-Asn-Pro-Gly-Val-Thr-Gln-Leu-Asn-Arg-Leu-Ala-Ala-His-Pro-Pro-Phe-Ala-Ser-Trp-Arg-Asn-Ser-Glu-Glu-Ala-Arg-Thr-Asp-Cys-Pro-Ser-Gln-Gln-Leu-[OH]

Composition : C259 H401 N81 082 S2
Weight : 6025.6872
C-Terminal : OH
N-Terminal : H
# of Residues : 55

Ac-Cys-Gly-Gly-Gly-Asp-Glu-Val-Asp-Gly-Ser-Leu-Ala-Val-Val-Leu-Gln-Arg-Arg-
-4 1 6
Asp-Trp-Glu-Asn-Pro-Gly-Val-Thr-Gln-Leu-Asn-Arg-Leu-Ala-Ala-His-Pro-Pro-Phe-Ala-Ser-Trp-Arg-

Asn-Ser-Glu-Glu-Ala-Arg-Thr-Asp-Cys-Pro-Ser-Gln-Gln-Leu
46 51

Chemistry : Fmoc
Composition:
Sequence : [H]-Cys-Lys-Gly-Gly-Gly-Ser-Thr-Pro-Pro-Thr-Pro-Ser-Pro-Ser-Ser-Leu-Ala-Val-Val-Leu-Gln-Arg-Arg-Asp-Trp-Glu-Asn-Pro-Gly-Val-Thr-Gln-Leu-Asn-Arg-Leu-Ala-Ala-His-Pro-Pro-Phe-Ala-Ser-Trp-Arg-Asn-Ser-Glu-Glu-Ala-Arg-Thr-Asp-Cys-Pro-Ser-Gln-Gln-Leu-[OH]

Composition : C279 H439 N87 089 S2
Weight : 6500.2474
C-Terminal : OH
N-Terminal : H
# of Residues : 60

Fig. 4

4	ω11 5	fx4 6	fx5 7	fx6 8	fx7 9
NO ENZ	8.554	63.30	26.67	228.8	377.5
0 DIL	109.9	428.5	375.1	563.6	518.4
1:10 DIL	14.24	375.1	269.7	472.8	496.1
1:100 DIL	118.0	96.57	49.11	267.6	399.4
1:1000 DIL	9.809	73.88	31.41	253.3	392.0
NO ED	0.363	0.363	0.382	0.377	0.412

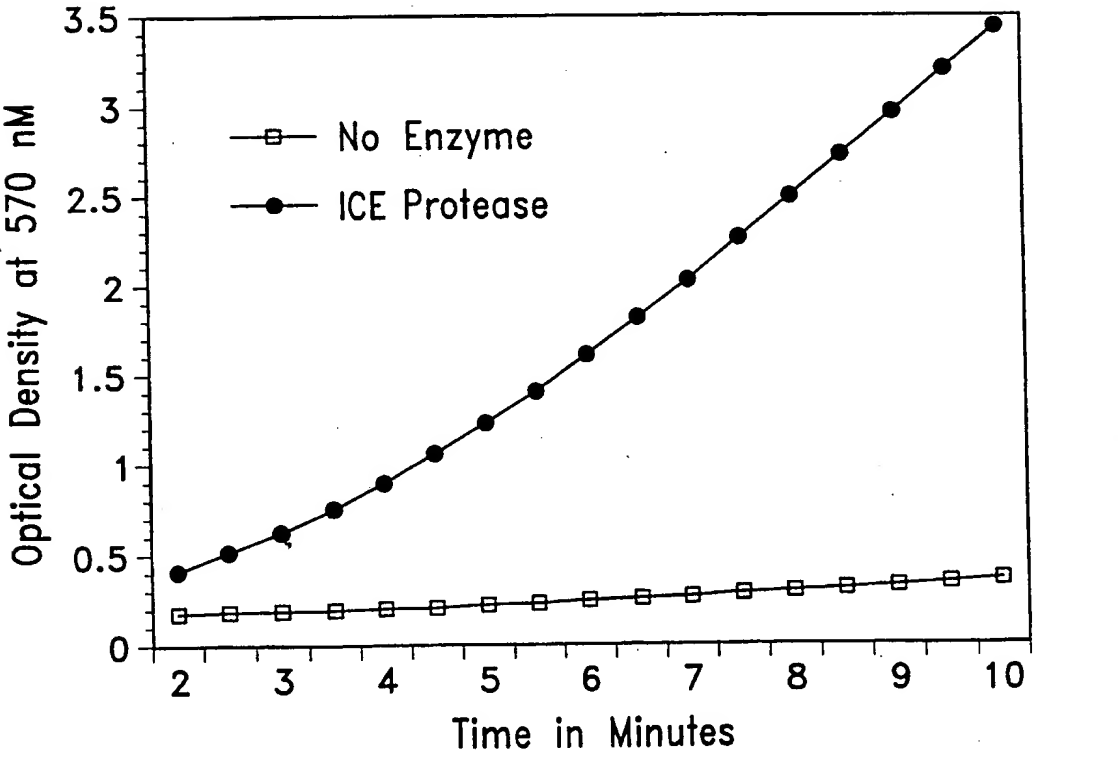


Fig. 5

H	Lys	Glu	Thr	Ala	Ala	Ala	Lys	Phe	Glu	Arg	Gln	His
	1	2	3	4	5	6	7	8	9	10	11	12
	Met	Asp	Ser	Ser	Thr	Ser	Ala	Ala	OH			
	13	14	15	16	17	18	19	20				

S-Peptide 1-20

H	Ser	Ser	Ser	Asn	Tyr	Cys	Asn	Gln	Met	Met	Lys	Ser
	21	22	23	24	25	26	27	28	29	30	31	32
	Arg	Asn	Leu	Thr	Lys	Asp	Arg	Cys	Lys	Pro	Val	Asn
	33	34	35	36	37	38	39	40	41	42	43	44
	Thr	Phe	Val	His	Glu	Ser	Leu	Ala	Asp	Val	Gln	Ala
	45	46	47	48	49	50	51	52	53	54	55	56
	Val	Cys	Ser	Gln	Lys	Asn	Val	Ala	Cys	Lys	Asn	Gly
	57	58	59	60	61	62	63	64	65	66	67	68
	Gln	Thr	Asn	Cys	Tyr	Gln	Ser	Tyr	Ser	Thr	Met	Ser
	69	70	71	72	73	74	75	76	77	78	79	80
	Ile	Thr	Asp	Cys	Arg	Glu	Thr	Gly	Ser	Ser	Lys	Tyr
	81	82	83	84	85	86	87	88	89	90	91	92
	Pro	Asn	Cys	Ala	Tyr	Lys	Thr	Thr	Gln	Ala	Asn	Lys
	93	94	95	96	97	98	99	100	101	102	103	104
	His	Ile	Ile	Val	Ala	Cys	Glu	Gly	Asn	Pro	Tyr	Val
	105	106	107	108	109	110	111	112	113	114	115	116
	Pro	Val	His	Phe	Asp	Ala	Ser	Val	OH			
	117	118	119	120	121	122	123	124				

S-Protein (21-124)

Fig. 6

Fab
Hinge
Fc
IgA1
IgA2
H
L
IgA1: -Pro-Ser-Thr-Pro-Pro-Thr-Pro-Ser-Pro-Ser-Thr-Pro-Pro-Thr-Pro-Ser-Pro-Ser-Cys-
225 230 235 240
IgA2: -Pro-Pro-Pro-Pro-Pro- (SEQUENCE DELETION) -Pro-Ser-Thr-Pro-Pro-Thr-Pro-Ser-Pro-Ser-Cys-
225 230 235 240
GalNac
Gal β1,3 GalNac
Gal β1,3 GalNac
CHO
S.sanguis
S.pneumoniae
H.influenzae
N.gonorrhosae
N.meningitidis-2
N.meningitidis-1
IgA protease cleavage sites

Fig. 7

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 98/15433

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K47/48 C12N9/38

According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	G. M. PAULETTI ET AL.: "Esterase-sensitive cyclic prodrugs of peptides: evaluation of an acyloxyalkoxy promoiety in model hexapeptide" PHARM. RES., vol. 13, no. 11, November 1996, pages 1615-1623, XP002082319	1,8,11, 13-15, 17,18
Y	*scheme 1* see page 1615	2-7,9,10

X	D. SHAN ET AL: "Prodrug strategies based on intramolecular cyclization reactions" J. PHARMACEUTICAL SCIENCES, vol. 86, no. 7, July 1997, pages 765-767, XP002082320 *schemes 5 and 6*	1

	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 October 1998	11/11/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Cervigni, S

1

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

ernational Application No
PCT/US 98/15433

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 89 11867 A (US GOVERNMENT) 14 December 1989 see page 7 - page 8; claims; figures 1-3 see page 13, paragraph 2	3-7
Y	US 5 646 298 A (POWELL MICHAEL J) 8 July 1997 see abstract see column 8, line 20, paragraph 60	2,9,10
P,X	WO 97 27320 A (BOEHRINGER MANNHEIM CORP ;POWELL MICHAEL J (US); PYARE KHANNA (US)) 31 July 1997 see the whole document	1-18
P,X	WO 97 27294 A (BOEHRINGER MANNHEIM CORP ;POWELL MICHAEL J (US); PYARE KHANNA (US)) 31 July 1997 see the whole document	1-18
P,X	WO 97 27203 A (BOEHRINGER MANNHEIM CORP ;POWELL MICHAEL J (US); TIETZE LUTZ F (DE) 31 July 1997 see the whole document	1-18
A	WO 92 03559 A (BOEHRINGER INGELHEIM INT) 5 March 1992 see page 10 - page 13	1-10
A	WO 95 12667 A (BOEHRINGER MANNHEIM CORP) 11 May 1995 see page 1-3	2,9,10

1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 98/ 15433

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claim 18 is directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

ernational Application No
PCT/US 98/15433

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 8911867 A	14-12-1989	US 5066490 A	19-11-1991
		AU 620417 B	20-02-1992
		AU 3768489 A	05-01-1990
		EP 0417188 A	20-03-1991
		JP 3502098 T	16-05-1991
US 5646298 A	08-07-1997	NONE	
WO 9727320 A	31-07-1997	US 5763196 A	09-06-1998
		AU 2111997 A	20-08-1997
WO 9727294 A	31-07-1997	AU 2245597 A	20-08-1997
		EP 0817840 A	14-01-1998
		JP 10507648 T	28-07-1998
WO 9727203 A	31-07-1997	AU 1837897 A	20-08-1997
		EP 0817789 A	14-01-1998
		JP 10508040 T	04-08-1998
WO 9203559 A	05-03-1992	DE 4027153 A	16-04-1992
		DE 4112952 A	22-10-1992
		CA 2072154 A	01-03-1992
		EP 0497949 A	12-08-1992
WO 9512667 A	11-05-1995	US 5492813 A	20-02-1996
		CA 2175061 A	11-05-1995
		EP 0736091 A	09-10-1996
		JP 9504695 T	13-05-1997

This Page is Inserted by IFW Indexing and Scanning Operations and is not part of the Official Record

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☐ FADED TEXT OR DRAWING
- ☐ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☐ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.